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Polymerase Resistance to Polymerase Chain Reaction Inhibitors in Bone*

ABSTRACT: Amplification of DNA from aged or degraded skeletal remains can be a challenging task, in part due to naturally occurring inhibitors of the polymerase chain reaction. PCR inhibitors may act by inactivating a polymerase itself, or compete with or bind other reaction components, although various polymerases may be differentially susceptible to such insult. In this study, ten thermostable polymerases from six bacterial species were examined for their ability to amplify DNA in the presence of bone-derived or individual PCR inhibitors. Two polymerases, one from *Thermus aquaticus* and one from *Thermus thermophilus*, showed lower susceptibility to inhibition from bone, while polymerases from *Thermus flavus* were highly susceptible. Addition of bovine serum albumin improved the activity of most of the enzymes. Taken together, the results indicate that thermostable DNA polymerases have different susceptibility to bone-derived PCR inhibitors, and that those most often used in forensic laboratories may not be optimal when working with DNA from skeletal remains.

KEYWORDS: forensic science, polymerase, polymerase chain reaction, inhibition, inhibitors, bovine serum albumin, *Thermus aquaticus*, *Thermus ubiquitous*, *Thermus flavus*, *Thermus litoralis*, *Thermus thermophilus*, *Thermus filiformis*, *Thermococcus gorgonarius*, *Thermococcus zilligii*, *Pyrococcus woesei*

The polymerase chain reaction (PCR), in which minute amounts of DNA are amplified into quantities that can readily be analyzed, has become a critical tool in forensic biology. Forensic samples are often not optimal sources of DNA however, and owing to any number of factors DNA amplification can fail. Assuming adequate amounts of DNA are present, the most common cause of PCR failure is PCR inhibition, wherein one or more undesirable components of a sample being tested are not eliminated during DNA purification, and subsequently interfere with amplification (1,2). These components may bind to or otherwise affect (e.g., degrade or compete with) the DNA itself, the primers, the nucleotides, Mg^{++}, or perhaps most importantly, negatively influence the DNA polymerase.

Many traditional DNA purification methods allow for the co-isolation of PCR-inhibitory substances. For example, phenol–chloroform extractions fail to remove water-soluble inhibitors such as urea or humic acids (3), Fe^{++} (4), and components of feces (5), while Chelex can be ineffective at removing inhibitors from blood samples (6,7). As such, alternative procedures have been utilized to increase DNA yield or overcome inhibition from particular biological mixtures (2,3,8,9). Additionally, extracted DNA may be repurified prior to PCR; methods include selective precipitation (10), silica membrane spin column filtration (7), Microcon filtration (11), gel filtration (12), DNA-binding silica beads (13), and immunomagnetic DNA separation (8).

All of these techniques can be effective at removing PCR inhibitors, however secondary purification usually results in a loss of DNA (14) as well as the time and expense of sample preparation (4). In cases involving aged and degraded samples where the amount of target DNA is already low, methods for improving amplification success without incurring DNA loss are limited to PCR optimization. The addition of chemical adjuvants to PCR can sometimes lead to improved amplification, either by optimizing PCR component interactions or combating inhibitors directly. Dimethyl sulfoxide (DMSO) has been shown to increase PCR and reverse transcriptase-PCR yields, presumably by stabilizing nucleic acid complexes and improving primer annealing efficiency (15,16). Kreader (4) demonstrated that the addition of bovine serum albumin (BSA) or T4 gene 32 protein could relieve PCR inhibition in the presence of 10–1000 times higher Fe^{++}, hemin, and humic substance levels than without, although neither improved inhibition from bilirubin, EDTA, NaCl, bile salts, or detergents.

A final method for overcoming PCR inhibition is to utilize a polymerase that is less susceptible to an inhibitor. Multiple researchers have observed that polymerases derived from different thermophilic bacterial species exhibit varying degrees of resistance to particular inhibitors. The most comprehensive study was an examination of potential inhibitors that make pathogen detection difficult in foodstuffs. Abu Al-Soud and Rådström (17) contrasted the ability of nine DNA polymerases to amplify bacterial DNA in the presence of complex biological mixtures, including blood, meat, cheese, and feces. In addition, polymerases were spiked with high levels of Mg^{++}, Ca^{++}, K^{+}, and Na^{+} to simulate ion concentrations that might be present in food samples. The authors demonstrated that AmpliTaq Gold (Applied Biosystems, Foster City, CA) and *Taq* (*Thermus aquaticus*) were more sensitive to Ca^{++} and inhibitors from blood than were *Pwo* (*Pyrococcus woesei*), *Tub* (*T. ubiquitous*), *Tfl* (*T. flavus*), and *Tli* (*T. litoralis*). Other researchers have compared the effect of PCR inhibitors on smaller numbers of polymerases. For instance, *Tth* (*T. thermophilus*) was found to retain polymerase activity in 5% phenol, whereas *Taq* was inhibited by a trace amount (18). *Tth* and *Tfl* were shown to be more resistant to inhibition than *Taq* in the presence of vitreous eye fluid

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(19). In a food pathogen detection assay, *Pwo* was more sensitive to inhibition than *Taq* in the presence of collagen (20). Unfortunately none of these studies was conducted in a forensic context, yet the relative inhibitor resistance of some polymerases suggests that the cognizant selection of a polymerase may be a useful tool in overcoming PCR inhibition encountered with DNA of forensic interest.

PCR-Suitable Thermostable Polymerases

DNA polymerases fall into distinct categories, which include families A, B, C, D, X, Y, and RT. The most extensively utilized replicative enzyme in PCR is *Taq* DNA polymerase, derived from the thermophilic bacterium *T. aquaticus. Taq* belongs to polymerase family A, the same family that includes the prokaryotic Pol I polymerase (21). Many commercial variants of *Taq* have been modified for "hot-start" functionality in order to increase amplification specificity. Additionally, other thermostable polymerases have been derived from thermophilic bacteria and archaea, which are commercially available in natural or recombinant forms. Most of these belong to families A (primarily bacterial repair polymerases) or B (primarily archaeal and eukaryotic replicative polymerases) (22), although recently there has been considerable effort to isolate thermostable Y-family polymerases for specialized PCR applications, namely the improved replication of UV-damaged DNA (23).

The enzymatic properties of polymerases (e.g., nuclease activity, fidelity, extension rates, and processivity—the average number of nucleotides added before disassociation of the enzyme) vary widely among families and species of origin (22), and commercial variants are often touted for one or more intrinsic properties that surpass *Taq*'s capabilities. For instance, *Pfu* (*P. fusorious*) reportedly exhibits a 12-fold higher fidelity rate than *Taq* (24). *Tth* DNA polymerase functions as both a polymerase and a reverse transcriptase (25), and may be utilized for one-tube reverse transcription PCR. Similarly, PCR strategies have been developed that use combinations of enzymes to overcome limitations of solo enzymes (26). Polymerase blends typically include a primary enzyme for DNA amplification, and a secondary polymerase that improves amplification efficiency (e.g., it adds a $3' \rightarrow 5'$ exonuclease, or "proofreading," function for reducing nucleotide misincorporation).

Finally, a large body of research has focused on reengineering thermostable polymerases to enhance fidelity and processivity or to confer additional functionality to the native enzyme. These modifications stem from either induced point mutations or genetic recombination. For example, random mutagenesis screens of *Taq* have resulted in cold-inactive variants for hot-start PCR applications (27). Site-directed mutagenesis has been utilized to create variants with lowered fidelity that can lead to purposefully mutagenic PCR (28) as well as mutants that more effectively incorporate ddNTPs for sequencing applications (29,30). Several DNA polymerase chimeras have been genetically engineered (23,31–33) that incorporate functional domains and structural motifs from native polymerases and other proteins to produce a "best of all worlds" construct. This is possible because the enzymes all have modular and independently folding domains, which allow for cooperative domain behavior despite different species origins (22). Most attempts to engineer chimeras have focused on improving commonly utilized PCR polymerases in order to enhance their natural capabilities. For instance, *Taq* was modified for increased processivity and fidelity through its fusion with the thioredoxin binding domain from T3 bacteriophage DNA polymerase (33). The processivity of both *Taq* and *Pfu* was greatly enhanced with the fusion of the double-stranded DNA binding protein, Sso7d, from the hyperthermophilic archaeabacteria *Sulfolobus solfataricus* (34).

Testing Alternative Thermostable Polymerase on Forensic Samples

As is apparent, a host of well-characterized, and often commercially available, thermostable polymerases exist that have not been explored by the forensic community, in spite of the difficulty that can be encountered when attempting to amplify DNAs of forensic interest. In part this is because the nature of forensic work requires observing standard operating procedures, which is not conducive to searching for new and improved reagents. Furthermore, many of the commercial kits utilized by forensic laboratories come with some form of *Taq* incorporated into their mix. Given these factors, the purpose of the current study was to assay a wide variety of polymerases on samples known to contain PCR inhibitors likely to be encountered in casework. Skeletal remains were focused upon as they often contain degraded and sub-optimal amounts of DNA (35), are known to have endogenous PCR inhibitors including Ca^{++} and collagen (36), and may also be recovered from environments that themselves contain PCR inhibitors (e.g., soil or water, with humic acids, fulvic acid, various metals, etc. [4,37]). Ten commercially available thermostable DNA polymerases—five variants of *Taq* polymerase and five from other bacteria—were tested for their ability to amplify nonhuman (porcine) bone DNA to which human bone DNA known to harbor high levels of PCR inhibitor(s) had been added. Additionally, the polymerases were tested for their ability to amplify human, nonskeletal DNA in increasing concentrations of exogenously added inhibitors (collagen, calcium, or humic acids) likely to be encountered with skeletal samples. Finally, PCR reactions were conducted with and without the addition of BSA in order to examine the adjuvant's alleviating effects on PCR inhibition, given the inhibitor and polymerase present.

Materials and Methods

Skeletal Material Processing and DNA Extraction

Two ancient human bones shown to exhibit high levels of PCR inhibition in previous analyses (38) were selected to serve as a source of inhibitors: a tibia fragment (from Diaporit, Albania, dated 5th–7th century AD) and a thoracic vertebra fragment (from Butrint, Albania, dated 5th–7th century AD). Nonhuman control DNA was obtained from a fresh porcine (*Sus scrofa*) ox coxae fragment. DNA extraction was performed in a Labconco (Kansas City, MO) Purifier PCR Enclosure. Tools were cleaned with 10% bleach and 70% ethanol and UV-irradiated for 10 min (*c.* 5 J/cm^2) prior to use.

A Dremel Rotary Tool (Dremel, Racine, WI) was used to sand away approximately 1 mm of surface bone. The newly exposed bone was swabbed twice with 10 mM EDTA, 0.5% SDS, 20 mM Tris pH 8, and allowed to air dry. The Dremel Tool was cleaned with 70% ethanol and fitted with a drill bit. A hole was drilled into the exposed bone surface to generate 200 mg powder, which was separated into two tubes. Three hundred microliters of digestion buffer (50 mM EDTA, 0.5% SDS, 20 mM Tris pH 8.0) and 5 μL of 20 mg/mL proteinase K were added to each tube. A reagent blank was also initiated. Samples and reagent blanks were vortexed for 15 sec and incubated at 55°C for 48 h.

Three hundred microliters of phenol was added, then tubes were vortexed for 30 sec and centrifuged at 14,000 rpm for 5 min. The aqueous layer was transferred to a new tube. Three hundred microliters of chloroform was added, then vortexed for 30 sec and

centrifuged at 14,000 rpm for 5 min. The aqueous layer was transferred to a Millipore (Billerica, MA) Microcon YM-100 centrifugal filter device and centrifuged at 500 × ***g*** for 12 min. Three hundred microliters of sterile water was added, then centrifuged at 500 × ***g*** for 15 min. Fifty microliters of sterile water was used for DNA recovery, after which the DNA was diluted to 1:5, 1:10, and 1:20 of the original concentration. DNAs were stored at −20°C.

Assayed Thermostable Polymerases

Five *Taq* polymerases from different commercial sources, as well as five non-*Taq* polymerases, were assayed. *Taq* varieties included two unmodified enzymes (Promega Corporation, Madison, WI and New England Biolabs, Ipswich, MA) as well as three modified for hot-start functionality: AmpliTaq Gold, HotMaster *Taq* (Eppendorf, Westbury, NY), and *Ex Taq* HS (Takara Bio, Shiga, Japan). Three other enzymes in the A family of polymerases, *Tfl* (Promega), *Tfi* (*Thermus filiformis*, Invitrogen, Carlsbad, CA), and *Tth* (Roche, Indianapolis, IN) were tested, as were two B family enzymes, *Tgo* (*Thermococcus gorgonarius*, Roche) and Pfx50 (*Thermococcus zilligii*, Invitrogen), that exhibit proofreading ability. Each enzyme was utilized with its supplied buffer; buffers accompanying *Tth* and Pfx50 contained BSA, while the others either did not contain the adjuvant or have proprietary formulae (HotMaster *Taq*, *Ex Taq* HS, and *Tfi*).

Bone Inhibitor PCR Assays

Ten microliter PCR reactions included 1× reaction buffer, 200 μM each dNTP, 1 U of polymerase, and 6 ng porcine DNA. If required, Mg^{++} was added to 2.5 mM. Porcine mtDNA primers targeting the ATPase gene (gcctaaatctcccctcaatggta and atgaaagaggcaaatagattttcg [39]) were at 2 μM, and generated a 212 base pair product. PCR reactions were spiked with either human tibia or vertebra inhibitor-containing DNA using 1 μL of the original extract or the 1:5, 1:10, or 1:20 dilutions. Positive and negative controls were included with every reaction set, and reactions were performed in duplicate. An additional vertebral replicate included 4 μg nonacetylated BSA (Fisher Biotech, Pittsburgh, PA). All PCR consisted of an initial 2 min incubation at 94°C (or as required for the enzyme) followed by 35 cycles of denaturing at 94°C for 30 sec, annealing primers at 53°C for 1 min, extension at 72°C for 30 sec, with a final incubation of 72°C for 7 min. Five microliters of PCR product was electrophoresed on a 2% agarose gel. PCR products were visualized with ethidium bromide staining and UV illumination.

Individual Inhibitor PCR Assays

Polymerase susceptibility to specific inhibitors, including collagen, Ca^{++}, and humic acids, were assayed in 10 μL volumes as described above with the following substitutions: 2 μM human mtDNA primers F15989 and R16410 (35) which generate a 421 base pair product, 2 ng human, nonskeletal DNA, 4 μg BSA, and 1 μL of inhibitor (see below). Type I collagen from calf skin (#C9791; Sigma-Aldrich Corporation, St. Louis, MO) was dissolved in 10 mM NaH_2PO_4 (adjusted to pH 3) and neutralized with 5 N NaOH to pH 7.0. Collagen was diluted with water to 1, 5, 10, 15, and 20 μg/μL. A control reaction contained 10 mM NaH_2PO_4 (also pH 7.0). Granular calcium chloride dihydrate (J. T. Baker, Phillipsburg, NJ) was dissolved in water to 10, 25, 50, 75, and 100 mM. Powdered humic acids (Alfa Aesar, Ward Hill, MA) were dissolved in water to 1, 10, 50, 100, and 1000 ng/μL. PCR consisted of an initial 2 min incubation at 94°C (or as required for the enzyme) followed by 35 cycles of denaturing at 94°C for 30 sec, annealing primers at 53°C for 1 min, and extending amplicons at 72°C for 1 min, with a final incubation of 72°C for 7 min. Positive and negative controls were included with every reaction set, and reactions were performed in duplicate. Reactions were visualized as described above.

Polymerase chain reaction products for each inhibitor and a subset of the polymerases were sequenced to confirm that amplicons were the intended human mtDNA region targeted. Sequencing was conducted using a CEQ DTCS Quick Start kit (Beckman Coulter, Fullerton, CA) in 10 μL volumes, following the manufacturer's protocol. Products were separated on a CEQ 8000 (Beckman Coulter), and sequences were compared to the known mitochondrial sequence of the control DNA.

Optimizing PCR Parameters

The various polymerases were assayed using human, non-skeletal DNA, and amplification conditions were optimized for each. The PCR conditions detailed above were suitable for DNA amplification with two exceptions: Pfx50 reactions required an increase in dNTP concentrations (400 μM), and conditions for *Tgo* were optimized with 20 ng DNA, 300 μM dNTPs, 3.75 mM Mg^{++}, and 0.1 U polymerase.

Results

DNA Amplification in the Presence of Bone Inhibitors

A PCR reaction was considered positive if a band could be visualized on an agarose gel following electrophoresis; however, in many instances DNA amplification success (band intensity) was a continuum, decreasing as inhibitor concentration increased (e.g., Fig. 1). Substantial differences were seen among polymerases when using a yes/no (band/no band) criterion (Table 1). *Tfl* failed to amplify the skeletally derived porcine DNA, even when no exogenous inhibitor was added. *Tfi* yielded very weak bands in the presence of the most dilute vertebra extract, and completely failed to amplify DNA in the presence of the tibia inhibitor. When assays were conducted using human, nonskeletal DNA, both enzymes generated an amplicon. *Taq* (NEB) was inhibited by the lowest concentrations of both inhibitors, while *Tgo* was inhibited by all but the most dilute solutions of both inhibitors. *Taq* (Promega) was resistant to all dilutions of the tibia inhibitor, but did not amplify in the presence of dilute vertebra inhibitor. Pfx50 amplified DNA in the highest concentration of tibia extract while being inhibited by the most dilute vertebra extract. The hot-start *Taq* variants amplified DNA at equal or higher inhibitor concentrations relative to unmodified *Taq.* Among these, *Ex Taq* HS was the least susceptible to the vertebra inhibitor, followed by AmpliTaq Gold and HotMaster *Taq*, while all were similarly resistant to the tibia inhibitor. *Tth* was the most inhibitor-resistant enzyme, and amplified DNA in the presence of the highest concentration of inhibitors from both sources.

Bovine serum albumin improved amplification for all enzymes in the presence of the vertebra inhibitor, with the exception of *Tfl*, which again did not amplify the porcine DNA even when no exogenous inhibitor was added. All enzymes except *Taq* (NEB), *Tfl*, and Pfx50 generated the target amplicon in the presence of undiluted inhibitor and BSA.

DNA Amplification in the Presence of Specific PCR Inhibitors

The results of assays that incorporated type I collagen, calcium, or humic acids as inhibitors are displayed in Table 2.

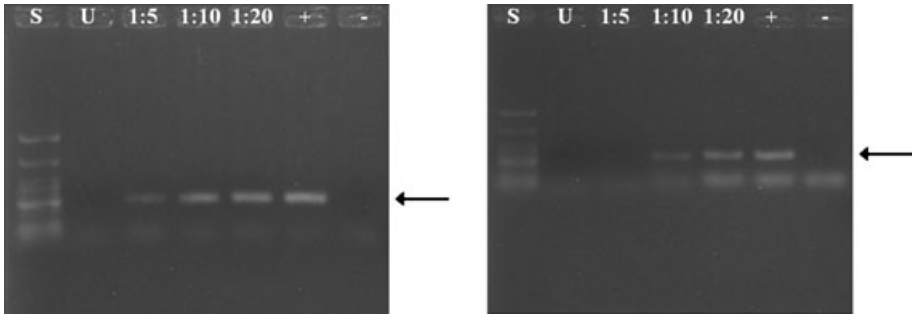

FIG. 1—*Examples of amplification results from PCR reactions spiked with inhibitors from ancient bone, highlighting the decrease in amplification efficiency as inhibitor concentration increased. (A) Represents dilutions of the tibia inhibitor amplified using AmpliTaq Gold. (B) Represents dilutions of the vertebra inhibitor amplified using HotMaster Taq. The arrows point to the 212 bp target amplicon. Lane "S" contains a size standard. Lane "U" shows PCR reactions spiked with undiluted bone inhibitors, while lanes "1:5", "1:10", and "1:20" were spiked with these extract dilutions. Lanes "+" and "−" are positive and negative controls.*

Polymerases/inhibitors generated the expected human mtDNA sequence. *Taq* variants exhibited different susceptibilities to collagen, but all amplified DNA when exposed to at least five times the previously reported minimum inhibitory concentration for *Taq* (0.08 μg/μL) (20). *Taq* (NEB) was the most susceptible to collagen inhibition, as no amplification was observed at 1 μg/μL, while *Taq* (Promega), AmpliTaq Gold, *Ex Taq* HS, *Tfi*, and Pfx50 became inhibited at 1.5 μg/μL. HotMaster *Taq*, *Tfl*, and *Tth* became inhibited at 2 μg/μL. *Tgo* was not inhibited at the highest concentration of collagen (2 μg/μL), although as noted in section Materials and Methods, this enzyme was optimized at 20 ng of template. The addition of BSA influenced only a subset of the enzymes; collagen-containing reactions with *Taq* (NEB and Promega), AmpliTaq Gold, HotMaster *Taq*, *Tth*, and *Tgo* did not improve when BSA was included, while *Ex Taq* HS, *Tfl*, *Tfi*, and Pfx50 were less inhibited.

TABLE 1—*DNA amplification results with bone-derived PCR inhibitors.*

Polymerase	None	1:20 Dilution	1:10 Dilution	1:5 Dilution	Neat
Tibia inhibitors					
Taq (NEB)	+/+	−/−	−/−	−/−	−/−
Taq (Promega)	+/+	+/+	+/+	−/+	−/−
AmpliTaq Gold	+/+	+/+	+/+	+/+	−/−
HotMaster *Taq*	+/+	+/+	+/+	+/+	−/−
Ex Taq HS	+/+	+/+	+/+	+/+	−/−
*Tfl**	−/−	−/−	−/−	−/−	−/−
*Tfi**	−/−	−/−	−/−	−/−	−/−
Tth	+/+	+/+	+/+	+/+	+/+
Tgo	+/+	+/+	−/−	−/−	−/−
Pfx50	+/+	+/+	+/+	+/+	+/+
Vertebra inhibitors					
Taq (NEB)	+/+	−/−	−/+	−/−	−/−
Taq (Promega)	+/+	−/−	−/−	−/−	−/−
AmpliTaq Gold	+/+	+/+	+/+	−/−	−/−
HotMaster *Taq*	+/+	+/+	−/+	−/−	−/−
Ex Taq HS	+/+	+/+	+/+	+/+	−/−
*Tfl**	−/−	−/−	−/−	−/−	−/−
Tfi	+/+	+/+	−/−	−/−	−/−
Tth	+/+	+/+	+/+	+/+	−/+
Tgo	+/+	−/+	−/−	−/−	−/−
Pfx50	+/+	−/−	−/−	−/−	−/−
Vertebra inhibitors—BSA added					
Taq (NEB)	+	+	+	+	−
Taq (Promega)	+	+	+	+	+
AmpliTaq Gold	+	+	+	+	+
HotMaster *Taq*	+	+	+	+	+
Ex Taq HS	+	+	+	+	+
*Tfl**	−	−	−	−	−
Tfi	+	+	+	+	+
Tth	+	+	+	+	+
Tgo	+	+	+	+	+
Pfx50	+	+	+	+	−

Inhibitory effects of DNA extracts from the human tibia and vertebra on DNA amplification with assayed polymerases. PCR reactions containing 6 ng of porcine DNA were spiked with 1 μL of neat bone inhibitor or a 1:5, 1:10, or 1:20 dilution. "+" denotes amplification product after 35 cycles, while "−" denotes no visible product after 35 cycles. Where indicated, 4 μg BSA was added to the reaction. Results are from two independent PCR reactions, except those with added BSA.

*Enzyme failed to amplify skeletally derived DNA when no inhibitor was added, but amplified human, nonskeletal DNA when no inhibitor was added.

The level of polymerase inhibition in the presence of added Ca^{++} varied considerably. *Tth* was inhibited at 2.5 mM Ca^{++}, *Taq* (NEB and Promega), AmpliTaq Gold, *Tfl*, *Tfi*, and Pfx50 were inhibited at 5 mM Ca^{++}, HotMaster *Taq* and *Ex Taq* HS were inhibited at 7.5 mM Ca^{++}, and *Tgo* was inhibited at 10 mM Ca^{++} (using 20 ng of template). The addition of BSA to the reactions resulted in increased inhibition of *Ex Taq* HS, but had no effect on DNA amplification for *Taq* (NEB and Promega), AmpliTaq Gold, *Tgo*, and Pfx50. *Tfl*, *Tfi*, *Tth*, and HotMaster *Taq* were less inhibited when BSA was included, with the latter showing the most appreciable improvement.

Taq (Promega), HotMaster *Taq*, and *Tfl* were inhibited at 1.0 ng/μL humic acids, while *Taq* (NEB), AmpliTaq Gold, *Ex Taq* HS, and *Tfi* were inhibited at 5.0 ng/μL humic acids, as was *Tgo* when attempting to amplify 20 ng of template. The remaining two polymerases, *Tth* and Pfx50, which have BSA in their buffers, exhibited the lowest susceptibility to humic acids, becoming inhibited at 10 and 100 ng/μL, respectively. Addition of BSA to the reactions enabled DNA amplification at 10 ng/μL humic acids for all polymerase tested, while they remained inhibited at the highest concentration of humic acids (100 ng/μL).

Discussion

The difficulties posed by PCR inhibitor-bearing DNA samples have traditionally been addressed by decreasing the inhibitor concentration, either through sample dilution or by further purifying the target DNA. Purification is effective when DNA copy number is sufficiently high, but the loss of target DNA that can occur with each cleanup step poses a fundamental problem with low copy material such as many skeletal remains. In contrast, PCR inhibition, at least in nonforensic samples, has been successfully overcome by utilizing a different polymerase (17–20). In the research presented here this concept was examined for PCR inhibitors of forensic interest, including specific ones (commercially available collagen, calcium, and humic acids) and those of less defined makeup,

TABLE 2—*DNA amplification results with individual PCR inhibitors.*

	Collagen									
	0.1 μg/μL		0.5 μg/μL		1.0 μg/μL		1.5 μg/μL		2.0 μg/μL	
Polymerase	Normal	BSA Added	Normal	BSA Added	Normal	BSA Added	Normal	BSA Added	Normal	BSA Added
Taq (NEB)	+/+	+/+	+/+	+/+	−/−	−/−	−/−	−/−	−/−	−/−
Taq (Promega)	+/+	+/+	+/+	+/+	+/−	+/−	−/−	−/−	−/−	−/−
AmpliTaq Gold	+/+	+/+	+/+	+/+	+/−	+/−	−/−	−/−	−/−	−/−
HotMaster *Taq*	+/+	+/+	+/+	+/+	+/+	+/+	+/+	+/+	−/−	−/−
Ex Taq HS	+/+	+/+	+/+	+/+	+/+	+/+	−/−	+/+	−/−	−/−
Tfl	+/+	+/+	+/+	+/+	+/−	+/+	+/−	+/−	−/−	+/−
Tfi	+/+	+/+	+/+	+/+	+/−	+/+	−/−	+/+	−/−	+/−
Tth	+/+	+/+	+/+	+/+	+/+	+/+	+/−	+/+	−/−	−/−
*Tgo**	+/+	+/−	+/+	+/−	+/+	+/+	+/+	+/+	+/+	+/−
Pfx50	+/−	+/−	+/+	+/+	+/+	+/+	−/−	+/−	−/−	−/−
	Calcium									
	1.0 mM		2.5 mM		5.0 mM		7.5 mM		10.0 mM	
Polymerase	Normal	BSA Added	Normal	BSA Added	Normal	BSA Added	Normal	BSA Added	Normal	BSA Added
Taq (NEB)	+/+	+/+	+/+	+/+	−/−	−/−	−/−	−/−	−/−	−/−
Taq (Promega)	+/+	+/+	+/+	+/+	−/−	−/−	−/−	−/−	−/−	−/−
AmpliTaq Gold	+/+	+/+	+/+	+/−	−/−	−/−	−/−	−/−	−/−	−/−
HotMaster *Taq*	+/+	+/+	+/+	+/+	+/+	+/+	−/−	+/+	−/−	+/−
Ex Taq HS	+/+	+/+	+/+	+/+	+/−	−/−	−/−	−/−	−/−	−/−
Tfl	+/+	+/+	+/+	+/+	−/−	+/−	−/−	−/−	−/−	−/−
Tfi	+/+	+/+	+/+	+/+	−/−	+/+	−/−	+/−	−/−	−/−
Tth	+/+	+/+	−/−	+/−	−/−	−/−	−/−	−/−	−/−	−/−
*Tgo**	+/+	+/−	+/+	+/+	+/+	+/+	+/+	+/−	−/−	−/−
Pfx50	+/+	+/−	+/−	+/−	−/−	−/−	−/−	−/−	−/−	−/−
	Humic acid									
	0.1 ng/μL		1.0 ng/μL		5.0 ng/μL		10.0 ng/μL		100.0 ng/μL	
Polymerase	Normal	BSA Added	Normal	BSA Added	Normal	BSA Added	Normal	BSA Added	Normal	BSA Added
Taq (NEB)	+/+	+/+	+/−	+/+	−/−	+/+	−/−	+/+	−/−	−/−
Taq (Promega)	+/+	+/+	−/−	+/+	−/−	+/+	−/−	+/−	−/−	−/−
AmpliTaq Gold	+/+	+/+	+/−	+/+	−/−	+/+	−/−	+/+	−/−	−/−
HotMaster *Taq*	+/+	+/+	−/−	+/+	−/−	+/+	−/−	+/+	−/−	−/−
Ex Taq HS	+/+	+/+	+/+	+/+	−/−	+/+	−/−	+/+	−/−	−/−
Tfl	+/+	+/+	−/−	+/+	−/−	+/+	−/−	+/+	−/−	−/−
Tfi	+/+	+/+	+/+	+/+	−/−	+/+	−/−	+/+	−/−	−/−
Tth	+/+	+/+	+/+	+/+	+/+	+/+	−/−	+/+	−/−	−/−
*Tgo**	+/+	+/+	+/+	+/+	−/−	+/+	−/−	+/+	−/−	−/−
Pfx50	+/+	+/+	+/+	+/+	+/+	+/+	+/+	+/+	−/−	−/−

Inhibitory effects of calcium, type I collagen, and humic acids on DNA amplification with assayed polymerases. PCR reactions containing 2 ng of human, nonskeletal DNA were spiked with 1 μL of inhibitor (calcium, collagen, or humic acids) to a final concentration as described in Materials and Methods. "+" denotes amplification after 35 cycles, while "−" denotes no visible product after 35 cycles. Where indicated, 4 μg BSA was added to the reaction. Results are from two independent PCR reactions.

*PCR reactions contained 20 ng DNA instead of 2 ng DNA.

originating from aged skeletal extracts previously shown to strongly inhibit PCR. Multiple thermostable polymerases were assayed, including *T. aquaticus* based enzymes widely used in forensic laboratories, as well as genetically modified enzymes and those from different thermophilic bacterial species.

In the research presented, introduction of PCR inhibitors derived from aged human skeletal remains acted as a relevant forensic assay of the polymerases, although it was important to know that negative results came from the inhibitors themselves, and not simply from a lack of DNA, which would clearly be a problem if the 1300–1600-year-old human bones acted as the only source of genetic material. To circumvent this, fresh bone DNA from a different species was assayed, ensuring that a lack of DNA was not a confounder during the study.

The influence of the human skeletal inhibitors on the different polymerases varied widely, although there were some general trends in polymerase performance. *Tth* and hot-start *Taq* variants (particularly *Ex Taq* HS) were resistant to higher concentrations of both skeletal inhibitors, while the remaining polymerases were active only at dilute inhibitor concentrations or not at all. Several polymerases were more inhibited by the vertebra extract than the tibia extract (with only *Tfi* showing the opposite trend), indicating differences in inhibitor concentration or composition between the two skeletal sources. One clue to the identity of inhibitors was the discoloration that the bones had assumed, presumably from soil exposure. The vertebra fragment and its extract had a reddish tint, which is characteristic of iron content (40,41), an established PCR inhibitor (4,17), presumably because iron competes with

polymerase/Mg^{++} interaction. The tibia fragment and its DNA extract were brown in color, perhaps indicative of soil-derived humic acids (42), thought to bind to *Taq* directly (43). If both iron and humic substances were present in the vertebra DNA extract, it could influence PCR via multiple mechanisms, helping to explain its more pronounced inhibition of the polymerases.

PCR inhibition by phenolic compounds such as humic acids is well documented (4,37,44–47). Humic acids are complex products of organic decomposition found in soil and water, and can be present in skeletal DNA extracts as a result of contact with these elements (4,45,48). BSA has been shown to relieve the PCR inhibitory effects of humic acids and iron (4), which may account for the lower bone inhibitor susceptibility observed with *Tth*, whose buffer contained BSA, versus polymerases that did not benefit from the adjuvant, as well as the general improvement observed with most polymerases when BSA was included. On the other hand, the poor amplification efficiency of Pfx50 and its BSA-containing buffer in the presence of the vertebra extract is puzzling, particularly considering that no inhibition of Pfx50 was observed with the tibia extract. This finding further indicates that the bone-derived inhibitors were affecting polymerase activity through multiple mechanisms. For instance, BSA is not highly effective at reducing inhibition caused by bilirubin and NaCl (4), likely because they have different modes of inhibition than do humic acids. If the vertebra extract contained multiple inhibitors, a polymerase less affected by such intangibles would be more apt to successfully amplify DNA.

In the current study, all polymerases were strongly inhibited by commercial humic acids unless BSA was present. In this regard, the humic acids results were most similar to those observed with the bone inhibitors, as DNA amplification of both was generally more successful when BSA was included, indicating the same mode of inhibition. The elimination of PCR inhibition afforded by BSA; that, was observed in the current research, and has been reported by others (4,49,50), makes the adjuvant a sensible addition to forensic PCR protocols involving skeletal samples, particularly if they have been exposed to soil. However, the effects of BSA on inhibited PCR appear to be concentration-limited. In one study (4), 400 ng/μL was determined to be the optimal BSA concentration for relieving PCR inhibition from humic acid, as additional BSA did not overcome inhibition of increasing humic acid; thus this concentration was used in the research presented here. Interestingly, two of the PCR buffers used in the current study already contained BSA; however, its concentration in the 1X *Tth* buffer was only 50 ng/μL, while the BSA concentration for Pfx50 was proprietary. PCR amplification efficiency for both enzymes was the same or improved when an extra 400 ng/μL BSA was present in the reaction. It is probable that BSA was originally included in these buffers for the purpose of relieving PCR inhibition, although the concentration could be raised considerably for maximum effectiveness.

Testing the various polymerases in the presence of the other defined inhibitors provided additional insight into the mechanisms through which they decrease PCR efficiency. Inhibition by type I collagen has been reported with food samples (20) and skeletal remains (36), although the mechanism of inhibition is undetermined. Kim et al. (20) observed that the effects of collagen on *Taq* and *Pwo* could be partially reversed with the addition of Mg^{++}, suggesting that collagen sequesters Mg^{++} or otherwise limits Mg^{++}/polymerase interaction. Given this, it seems plausible that other polymerases might amplify DNA in the presence of collagen through the addition of more Mg^{++}, although this was not explored in the current research.

Polymerase chain reaction inhibition due to calcium ions has also been reported in food science research (17,51) as well as clinical studies (52), and is thought to occur via competitive exclusion of Mg^{++} (51). The data presented here support the proposed mechanism, as Pfx50 and *Tth* PCR buffers contained the lowest concentrations of Mg^{++} (1.2 and 1.5 mM final concentration, respectively), and both proved to be the most susceptible to Ca^{++} inhibition, while *Tgo*, with the highest Mg^{++} concentration (3.75 mM), was the most resistant. This suggests that Ca^{++} inhibition, like collagen inhibition, might be overcome by increasing Mg^{++} in the PCR mixture, although polymerase choice might be limited to those enzymes insensitive to high Mg^{++} concentrations, which some, including *Tfl*, *Taq*, and AmpliTaq Gold, are not (19,51).

Conclusions

Polymerase chain reaction inhibitor resistance is an important property to consider when selecting a polymerase for DNA amplification, particularly when samples likely to harbor inhibitors are examined, as is often the case with forensic material. Of the enzymes tested, *Tfl*, *Tfi*, *Tgo*, and Pfx50 were most susceptible to skeletal inhibitors, with *Tfl* inhibited even by fresh bone, and thus none appear to be good candidates for skeletal DNA analyses. Hot-start *Taq* variants performed better in the presence of skeletal inhibitors than did standard *Taq*, with *Ex Taq* HS leading over AmpliTaq Gold and HotMaster *Taq*. Finally, *Tth* consistently outperformed all other polymerases in high concentrations of skeletal PCR inhibitors; thus it, along with *Ex Taq* HS, appear most advantageous for assaying skeletal remains.

It should be noted that BSA improved amplification success for almost all of the enzymes in the presence of the skeletal inhibitors, and is a worthwhile adjuvant in general. Therefore, polymerases that are prepackaged with BSA in their buffers may be more suitable for overcoming skeletally derived inhibitors relative to products that lack the adjuvant. However, buffer BSA levels are not necessarily optimal for use on skeletal DNAs, thus addition of BSA to at least 400 ng/μL should be considered for maximal inhibitor relief.

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